A photoautotrophic *in vitro* system for evaluating salt tolerance of soybean (*Glycine max* L.) plants

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Abstract

To explore the possibility of employing the photoautotrophic *in vitro* system for evaluating salt tolerance of soybean cultivars, a study was conducted. Three soybean cultivars, 'Lee 68', 'Zhonghuang 13', and 'Union' were cultured photoautotrophically (sugar-free medium) *in vitro* under seven water potentials. After three weeks, plantlet growth and leaf chlorophyll, proline, and soluble sugar contents showed that salt tolerance of these cultivars increased in the order of 'Union', 'Zhonghuang 13', and 'Lee 68' with abilities to tolerate water potentials up to –0.45, –0.91, and –1.14 MPa, respectively. The photoautotrophic *in vitro* salt tolerance evaluating system is apparently faster, efficient, and saves labour and cost as the performance of the cultivars can be evaluated in a few weeks compared to several months in the conventional field or greenhouse evaluations.

Keywords: Proline, Soluble sugar, Salt stress.

Introduction

Soybean (*Glycine max* L.) productivity is inhibited in the saline soils (Essa and Al-Ani, 2001). One of the effective ways that soybean plants adapt to salt stress is to accumulate the compatible solutes, such as proline and soluble sugars. Salt tolerant soybean plants possess a high capacity for biosynthesis and accumulation of these compatible solutes to resist osmotic stress (Parida and Das, 2005). Thus, an effective approach for minimizing soybean yield loss in saline soil is to employ cultivars with high salt tolerance. However, conventional methods for evaluating the salt tolerance of soybean cultivars in the field or greenhouses require much time, space, and labour. Tissue culture, which overcomes such problems, may be an effective method for evaluating the salt tolerance of soybean cultivars. However, in conventional tissue culture using sugar containing medium, the plantlets' photosynthetic ability is inhibited and they grow hetero- or photomixotrophically, using sucrose in the medium as the main source of carbohydrates. The results of conventional tissue culture thus cannot match the results of field cultivation, where the plants grow photoautotrophically (Cha-um et al., 2005).

A photoautotrophic *in vitro* system might be an efficient means for evaluating the salt tolerance of soybean cultivars. Because the green, leafy plantlets grow photoautotrophically *in vitro* on a sugar-free medium by properly controlling the *in vitro* physical environmental factors, such as light, CO_2, temperature, and humidity. Moreover, a larger, force-ventilated and/or CO_2 enriched culture vessel containing sugar-free medium is used, which acts like a miniature greenhouse (Kozai et al., 2005). The photoautotrophic *in vitro* system has been successfully used for assessing salt stress response in *Albizzia lebbek* and for salt tolerance screening of rice and about 100 forest tree species (Cha-um et al., 2004). The objective of this study was to explore the efficiency and possibility of employing the photoautotrophic *in vitro* system for evaluating salt tolerance of soybean cultivars.

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Materials and Methods

The experiment was conducted using three cultivars of soybean plants: a relatively salt tolerant cultivar 'Lee 68', a common cultivar in China 'Zhonghuang 13', and a relatively salt sensitive cultivar 'Union' (Shao et al., 1995; Essa and Al-Ani, 2001). The seedling stage which is the most sensitive stage to salt was used in these trials. Soybean plantlets were established from seeds germinated aseptically (surface sterilized in Cl_2 for 16 h) on sugar-free MS medium (brands of agar, 8 $g \cdot L^{-1}$). Single node cuttings (85 mg and 3 cm long) excised from the plantlets were used as explants. The explants were cultured photoautotrophically (CO_2 as a carbon source) for three weeks. The Murashige and Skoog gelled (brands of agar, 8 $g \cdot L^{-1}$) medium excluding glycin and including 1 $mg \cdot L^{-1}$ IBA was used as the culture medium. Salt stress, induced by lowered water potential was generated by increasing the NaCl content of the culture medium. The culture media with seven different water potentials obtained by adjusting the content of NaCl are described in Table 1. The culture vessels were Magenta-type vessels (polycarbonate, 370 ml) containing the medium (70 ml in each vessel) with two gas permeable filter disks (10 mm diameter; pore size: 0.5 µm, Milliseal, Millipore, Tokyo) on the lid. Ten vessels (two explants in each vessel) were used in each treatment. Each treatment was replicated three times. The culture room was maintained 25±1°C, 65±5% relative humidity and 800±50 $\mu mol \cdot mol^{-1}$ CO_2 concentration using a web-server embedded environment control system. White fluorescent lamps provided a photosynthetic photon flux density (PPFD) of 70±9 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with 16 $h \cdot d^{-1}$ photoperiod.

The plantlet growth and chlorophyll content in the soybean leaves expressing plant damage caused by salt stress or low water potential and the contents of proline and soluble sugar in the soybean leaves expressing salt tolerance were evaluated. At the end of the 21-day culture period, five vessels were randomly harvested in each treatment and the fresh weights of plantlets were measured. Dry weights of plantlets were measured after drying at 80°C for 72 h. Root growth and chlorosis of the plantlets were observed during the experiment. Chlorophyll content in leaf tissue was estimated as described by Arnon (1949). For extracting chlorophyll in leaf tissue, fresh leaf (0.8 g) was soaked in a test tube with 10 ml solvent consisting of equal volumes of anhydrous ethanol and 80% acetone. Absorbance of the extracted solution was measured at wavelengths of 645 and 663 nm using the spectrophotometer (UV3150, Shimadzu Corporation, Japan). Chlorophyll *a* and *b* contents were calculated according to the following equations and summed up.

$[Chl_a] = 12.7\, A_{663} - 2.69\, A_{645}$

$[Chl_b] = 22.9\, A_{645} - 4.68 A_{663}$

where A_i is the optical density at the wavelength of i.

Proline content in leaf tissue was determined by a modified method described by Bates et al. (1973). Fresh leaf (0.2 g) was homogenized in a test tube with 5 ml of 3% sulfosalicylic acid. The test tube was placed in boiling water for 10 min. From the test tube, two ml of the

Table 1. Water potentials[1] of the culture medium.

NaCl concentration ($mMol \cdot L^{-1}$)	Water potential caused by NaCl (MPa)	Total water potential (MPa)
0	0	–0.22
51	–0.23	–0.45
103	–0.46	–0.68
154	–0.69	–0.91
206	–0.92	–1.14
257	–1.14	–1.36
309	–1.37	–1.59

[1] Total water potential was defined as the sum of water potentials caused by MS medium and sodium chloride. The water potential of the MS medium is –0.22 MPa (Kozai et al., 1986) and the estimated values were calculated based on Jones (1992).

supernatant was added to 2 ml glacial acetic acid and 3 ml of acid-ninhydrin (a mixture of 1.25 g of ninhydrin, 30 ml glacial acetic acid, and 20 ml 6 M phosphoric acid). The tubes were maintained in boiling water for 40 min and after cooling to room temperature, 5 ml toluene was added and the layers were allowed to partition. Absorbance of the toluene layer was measured at 520 nm using a spectrophotometer (UV3150). The proline content was determined from a standard curve of 0 to 20 µg L-proline.

Soluble sugar content of leaf tissue was evaluated by a modified method reported by Dubois et al. (1956). Fresh leaf (0.3 g) was homogenized in a test tube with 5 to 10 ml distilled water and placed in a boiling water bath for 30 min. From the homogenized solution, residues of the leaf tissues were removed. Volume of the homogenized solution was adjusted to 25 ml by adding distilled water. To 0.5 ml of the homogenized solution, 1.5 ml distilled water, 1 ml 9% phenol and 5 ml sulfuric acid were added and the tubes were kept at room temperature for 30 min. Absorbance was measured at a wavelength of 485 nm using a spectrophotometer (UV3150). The soluble sugar content was calculated from a standard curve of 0 to 100 µg of sucrose.

Statistical differences among the treatments of the same cultivar were analyzed by the least significant difference (LSD) test ($p<0.05$) when analysis of variance (ANOVA) by SPSS software (SPSS for Windows, SPSS Inc., USA) indicated treatment significance.

Results and Discussion

Despite salt tolerance, dry weight of the soybean plants was inhibited by salt or water stress (Table 2). Soybean cultivars responded differently to increasing salt stress or decreasing water potentials. Less reduction was shown in dry weight of the relatively salt tolerant cultivar 'Lee 68' than that of the relatively salt sensitive cultivar 'Union'. No root initiation was observed when the water potential of the medium was lower than –0.68 MPa for 'Lee 68', or –0.45 MPa for 'Zhonghuang 13' and 'Union'. Essa and Al-Ani (2001) also observed that despite the salt tolerance of the soybean plants, root initiation was significantly inhibited by high salt stress.

Chlorophyll content in the leaves of the three soybean cultivars significantly decreased with decreasing water potential (Fig. 1). It is well known that chlorophyll content is sensitive to environmental stresses and it is often used as an index of the damage caused by adverse conditions. Salt stress not only suppresses biosynthesis of chlorophyll, but also accelerates its degradation (Shao et al., 1995). Chlorosis, one of the phenotypic expressions of plants' chlorophyll content under environmental stress, observed at the 10th day in 'Lee 68' and at the 7th day in 'Zhonghuang 13' and 'Union' also indicated that leaves of a relatively salt sensitive soybean cultivar 'Union' appeared chlorotic earlier than others and the problem was more severe with increasing exposure time and decreasing water potentials. This is consistent with the results of Cha-um et al. (2005).

Table 2. Fresh weights of soybean plantlets cultured photoautotrophically (sugar-free medium) *in vitro* under seven water potentials (21days after culturing).

Water potential (MPa)[1]	Stem and leaf (mg)			Root (mg)		
	'Lee 68'	'Zhonghuang 13'	'Union'	'Lee 68'	'Zhonghuang 13'	'Union'
–0.22	595 ± 58.0a	563 ± 75.2a	546 ± 41.7a	415. ± 99.4a	412. ± 13.6a	243. ± 90.5a
–0.45	450 ± 42.7b	337 ± 27.4b	260 ± 40.8b	100. ± 18.9b	70.1 ± 16.9b	59.5 ± 19.0b
–0.68	332 ± 34.7c	254 ± 18.3bc	193 ± 25.7bc	41.1 ± 8.20b	–	–
–0.91	225 ± 16.3d	180 ± 21.3cd	175 ± 16.3bc	–[2]	–	–
–1.14	197 ± 15.7de	138 ± 24.1d	132 ± 13.3bc	–	–	–
–1.36	110 ± 4.10 e	133 ± 18.8d	134 ± 39.9c	–	–	–
–1.59	109 ± 6.00 e	131 ± 24.4d	135 ± 43.9c	–	–	–

[1]See Table 1.; [2]No data.; Means followed by the same alphabet are not significantly different ($p<0.05$).

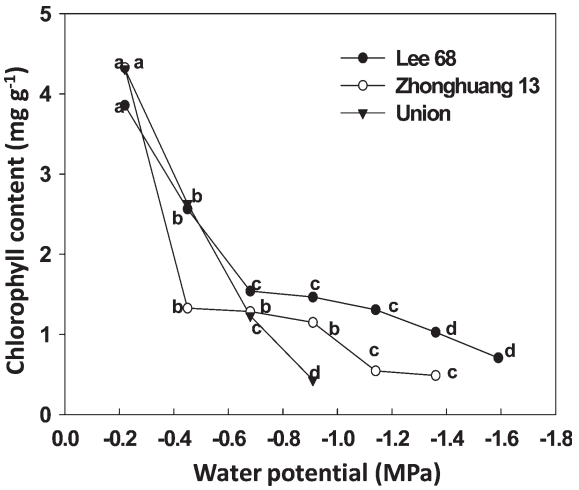

Figure 1. Chlorophyll concentrations of soybean plantlets cultured photoautotrophically (sugar-free medium) *in vitro* under seven water potentials (21days after culturing). Data points with the same alphabet are not significantly different (p<0.05).

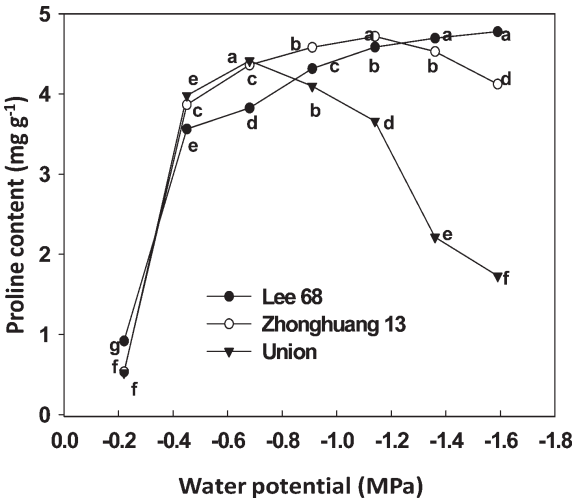

Figure 2. Foliar proline concentrations of soybean plantlets cultured photoautotrophically (sugar-free medium) *in vitro* under seven water potentials (21days after culturing). Data points with the same alphabet are not significantly different (p<0.05).

We also noted significant accumulations of proline at high NaCl concentrations (strongly negative water potentials) in the medium for all cultivars (Fig. 2). 'Lee 68', the relatively salt tolerant cultivar, however, had a higher foliar proline concentration than the other two cultivars at high stress levels (–1.2 to –1.6 MPa). Nonetheless, this cultivar had relatively lower foliar proline concentrations than the other two cultivars at intermediate water potentials. Implicit in this is that the ability for biosynthesis and accumulation of proline was higher in plants with high salt tolerance, especially when subjected to intense stress. Although the mechanism of regulation of proline biosynthesis in higher plants is still unclear, the role of proline in plant adaptation to water deficit and salinity stress is well known and it acts as a non-toxic compatible or counteracting organic solute (Parida and Das, 2005). Proline accumulation responds to salt stress via an osmoregulation system that consumes much energy. As can be seen from Fig. 2, the foliar proline concentrations of both 'Zhonghuang 13' and 'Union' increased at higher water potentials and decreased at lower water potentials. But the foliar proline concentrations of 'Lee 68' gradually increased with decreasing water potential, implying its higher relative salt tolerance.

Soluble sugar concentrations in the leaves of the relatively salt sensitive cultivar 'Union' changed hardly with decreasing water potential, indicating their sensitivity to salt stress (Fig. 3). Presumably, these plants cannot produce more soluble sugar to adapt to

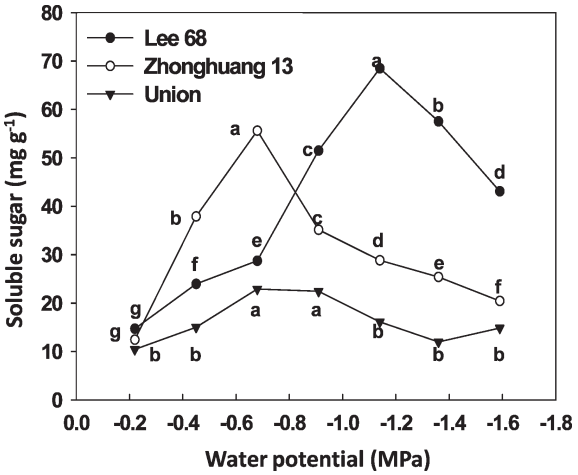

Figure 3. Foliar soluble sugar concentrations of soybean plantlets cultured photoautotrophically (sugar-free medium) *in vitro* under seven water potentials (21days after culturing). Data points with the same alphabet are not significantly different (p<0.05).

salt stress. Contrarily, 'Lee 68' and 'Zhonghuang 13' plants produced soluble sugar to adapt to salt stress, indicating higher salt tolerance. The accumulation of soluble sugar in leaves under salt stress not only functions as an osmoregulant but also plays a role in energy preservation. Once the plant is released from the episode of salt stress, these soluble sugar could be its main carbon source (Zhifang and Loescher, 2003). Accumulation of soluble sugars also depends on photosynthesis to supply the appropriate substrates and in turn protects photosynthetic metabolism (Essa and Al-Ani, 2001; Parida and Das, 2005).

In this experiment, the photoautotrophic *in vitro* system was evaluated as a tool for monitoring the salt tolerance of soybean cultivars. Results indicate that salt tolerance of the soybean cultivars evaluated increased in the order of 'Union', 'Zhonghuang 13' and 'Lee 68' with abilities to tolerate water potentials up to –0.45, –0.91, and –1.14 MPa, respectively. The photoautotrophic *in vitro* system can be considered as an efficient method for evaluating the salt tolerance of soybean plants, and to some extent, may even obviate the need for field or greenhouse experimentations for this purpose. As can be seen from our results, it is possible to evaluate salt tolerance of the cultivars within a relatively short period of time (about a month). In the conventional field and greenhouses experiments, however, this takes much longer. Other advantages of employing the photoautotrophic *in vitro* system to evaluate the salt tolerance of soybean plants are cost reduction, obviously due to the shorter period of experimentation under laboratory conditions as opposed to the much longer period of experimentation required under field or greenhouse conditions. Moreover, it is possible to simulate variable environmental regimes in the photoautotrophic *in vitro* system, but such procedures may be very cumbersome in the field studies.

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